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Award Number: DAMD17-01-1-0582

TITLE: High Density Lipoprotein Complexes as Delivery Vehicles for Breast Cancer Chemotherapy

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REPORT DATE: May 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2002	Annual (1 May 01 - 30 Apr 02)

4. TITLE AND SUBTITLE
High Density Lipoprotein Complexes as Delivery Vehicles for Breast Cancer Chemotherapy

5. FUNDING NUMBERS
DAMD17-01-1-0582

6. AUTHOR(S)
Andras G. Lacko, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Reconstituted high density lipoprotein (rHDL) particles have been developed to incorporate water insoluble drugs to serve as drug delivery agents. Specifically, we have prepared taxol (TX) containing rHDL particles that are stable on rechromatography and preparative ultracentrifugation as no losses of TX from the complexes were seen during these studies. The rHDL/ TX complexes were as toxic to breast cancer cells as the commercial cremophor/taxol preparations. Taxol was avidly taken up by cancer cells from the rHDL/TX complexes, apparently by an SRB1 type receptor mediated mechanism. The uptake of core components from the rHDL complexes by a receptor mediated mechanism is supported by the observed expression of the SRB1 type receptors (demonstrated by immunoblotting) and by competition studies. Cancer cells were shown to exhibit considerably higher SRB1 receptor expression than fibroblasts, suggesting that the rHDL drug delivery system may be specifically targeting malignant cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, rHDL, SRB1 receptor	25
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Adjuvant cancer chemotherapy has long been the accepted strategy for the treatment of several types of malignant tumors, especially those involving metastases (1). To enhance the efficacy of chemotherapy, multiple agents, longer and more intensive treatment, and combination therapy have been used. Although these modified approaches have resulted in improvements, the severity of side effects (2) and the gradual resistance developed by tumors during therapy (3) continue to hinder the efficacy of cancer chemotherapy. Consequently, more efficient delivery systems are needed to reduce the toxicity associated with cancer chemotherapy, while preserving the effectiveness of the respective chemotherapeutic agents.

In order to reduce the toxicity of chemotherapeutic agents, delivery vehicles including liposomes have been investigated (4,5). Some of the data emerging from these studies have shown that the efficacy of the drug delivery by liposomes was inversely related to the diameter of the particle (6). However, even the smallest liposomes have a diameter five times larger than the average high density lipoprotein (HDL) particle, a hydrophobic lipid micelle core with surface components of phospholipid and apolipoproteins (7). Because the overall properties of HDL are consistent with a potentially superior hydrophobic drug transporter, we have undertaken the investigation of rHDL particles as delivery vehicles for cancer chemotherapeutic agents.

While preparations of reconstituted HDL were developed over thirty years ago (8), this approach has been used primarily to characterize the physical and chemical properties of HDL (9-11) and to conduct metabolic studies (12-13). Trials aimed at a clinical application of HDL particles have been reported to relieve endotoxic shock (14) and to arrest or reverse atherosclerosis (15). An application involving reconstituted HDL has been reported by Bijsterbosch and Van Berkel (16) who used lactosylated HDL to target delivery of antiviral drugs to liver tissue. Earlier studies also indicated that the proliferation of breast cancer cells and other adenocarcinoma cells (17-19) are dependent on the uptake of HDL components. Thus an additional advantage of using HDL for delivering chemotherapeutic agents is the direct uptake of hydrophobic core components from HDL by specific cell surface receptors, including those expressed by tumor cells (20,21).

The present study shows that a number of cultured human cancer cells are nearly as efficient as the model ovarian granulosa cells (HGL5) in the uptake of cholesteryl esters and taxol from the lipid core of recombinant HDL (rHDL) complexes, indicating that HDL may be used to solubilize otherwise very poorly water soluble (hydrophobic) therapeutic agents for injection into the systemic circulation.

KEY RESEARCH ACCOMPLISHMENTS

This is a "Concept Award" report. Due to the limited funding, the accomplishments are correspondingly modest. Nevertheless, we are pleased to report numerous exciting findings, a paper accepted to be published in "Anticancer research" and some extended funding for this project.

The following scientific accomplishments can be documented during the first year of this award.

1. Reconstituted high density lipoprotein (rHDL) particles have been developed that are able to incorporate water insoluble drugs as core components and thus are excellent potential targeted drug delivery agents.

2. Specifically, we have prepared taxol (TX) containing rHDL particles that exhibit the expected molecular weight, lipid and protein compositions(similar to that of human HDL.

3. These rHDL/TX particles are stable on rechromatography and preparative ultracentrifugation as no losses of TX from the complexes were seen during these studies.

4. The rHDL/ TX complexes were as toxic to breast cancer cells as the commercial cremophor/taxol preparations. Taxol was avidly taken up by cancer cells from the rHDL/TX complexes, apparently by an SRB1 type receptor mediated mechanism.

5. The uptake of core components from the rHDL complexes by a receptor mediated mechanism is supported by the observed expression of the SRB1 type receptors (demonstrated by immunoblotting) and by competition studies. Cancer cells were shown to exhibit considerably higher SRB1 receptor expression than fibroblasts, suggesting that the rHDL drug delivery system may be specifically targeting malignant cells.

These studies show that the rHDL drug delivery system is effective in killing breast cancer cells while it may have reduced toxicity toward normal cells. The ability of the rHDL delivery vehicles to solubilize otherwise highly hydrophobic drugs (taxol) without the use of toxic solubilizing agents (cremophor) is an additional advantage of this sytem. The rHDL particles may also be able to overcome the drug resistance of many tumors because of the receptor mediated uptake of their core components.

Studies in Progress:

We are currently investigating the stability of the HDL/drug complexes in detail. We have identified *dilauroyl fluoresceine* as an ideal model compound for these studies. We are assessing the in vivo stability of the HDL/drug complexes by injecting these preparations into mice. Finally, we have initiated studies with alternate processes for producing reconstituted HDL particles in addition to exploring the drug delivery potential of intact, circulating HDL particles.

REPORTABLE OUTCOMES

We have had an article accepted to be published in "Anticancer Research" (see attached).

We are presenting our findings at the "ERA of Hope" meeting in September, 2002.

We have obtained additional pilot funding to continue this project from the Institute for Cancer Research and from the Bank One cancer Research Fund.

We have filed an application with the US Patent Office.

CONCLUSIONS

We have completed al the projected goals that were outlined for this project in the "Concept proposal". The next phase of our studies involves translational studies with animals that will require substantially higher level of funding. Accordingly, we have been submitting proposals to the DOD Breast Cancer Program, the National Institutes of Health and to the Susan G. Komen Breast Cancer Foundation. We are hopeful that these efforts will result in additional funding and the continuation of our work.

Regarding the overall potential impact of our research, we believe that it has unlimited potential. Accordingly, our research has wide applications in breast cancer chemotherapy because, in addition to taxol, many of the frequently employed drugs are poorly water soluble and their application would thus be substantially improved by the rHDL delivery system. In addition, because of the vast potential for targeting, via modifications of its protein or lipid components, the future application of the rHDL drug delivery system could revolutionize chemotherapy via tumor specific targeting and potentially overcoming drug resistance. The proposed approach has the additional potential to substantially improve the delivery of anticancer drugs to hormone resistant breast tumors and thus enhance the prognosis for the survival of breast cancer patients.

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APPENDICES:

Paper accepted by Anticancer Research.

High density lipoprotein complexes as delivery vehicles for anticancer drugs

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ABSTRACT

Materials and Methods: Recombinant high density lipoprotein (rHDL) particles were prepared with defined composition (phosphatidylcholine, apolipoprotein AI, cholesterol and cholesteryl esters) and a molecular weight of approximately 187,000 kdaltons. Three molecules of taxol per rHDL particle were incorporated into these rHDL complexes.

Results: Cholesteryl ester and taxol (HDL core components,) were taken up efficiently by several cancer cell lines compared to transformed normal ovarian cells (HGL5) used as the control. Immunoblotting with the scavenger receptor (SR-B1) antibody revealed strong immunoreactivity of several cancer cell lines.

Conclusion: Our studies indicate that rHDL complexes can be used as efficient drug delivery vehicles due to the ability of cancer cells to acquire HDL core components. Targeting of anti-cancer drugs as components of the rHDL complex could result in reduction of toxic side effects during chemotherapy.

INTRODUCTION

Adjuvant cancer chemotherapy has long been the accepted strategy for the treatment of several types of malignant tumors, especially those involving metastases (1). To enhance the efficacy of chemotherapy, multiple agents, longer and more intensive treatment, and combination therapy have been used. Although these modified approaches have resulted in improvements, the severity of side effects (2) and the gradual resistance developed by tumors during therapy (3)

continue to hinder the efficacy of cancer chemotherapy. Consequently, more efficient delivery systems are needed to reduce the toxicity associated with cancer chemotherapy, while preserving the effectiveness of the respective chemotherapeutic agents.

In order to reduce the toxicity of chemotherapeutic agents, delivery vehicles including liposomes have been investigated (4,5). Some of the data emerging from these studies have shown that the efficacy of the drug delivery by liposomes was inversely related to the diameter of the particle (6). However, even the smallest liposomes have a diameter five times larger than the average high density lipoprotein (HDL) particle, a hydrophobic lipid micelle core with surface components of phospholipid and apolipoproteins (7). Because the overall properties of HDL are consistent with a potentially superior hydrophobic drug transporter, we have undertaken the investigation of rHDL particles as delivery vehicles for cancer chemotherapeutic agents.

While preparations of reconstituted HDL were developed over thirty years ago (8), this approach has been used primarily to characterize the physical and chemical properties of HDL (9-11) and to conduct metabolic studies (12-13). Trials aimed at a clinical application of HDL particles have been reported to relieve endotoxic shock (14) and to arrest or reverse atherosclerosis (15). An application involving reconstituted HDL has been reported by Bijsterbosch and Van Berkel (16) who used lactosylated HDL to target delivery of antiviral drugs to liver tissue. Earlier studies also indicated that the proliferation of breast cancer cells and other adenocarcinoma cells (17-19) are dependent on the uptake of HDL components. Thus an additional advantage of using HDL for delivering chemotherapeutic agents is the direct uptake of

hydrophobic core components from HDL by specific cell surface receptors, including those expressed by tumor cells (20,21).

The present study shows that a number of cultured human cancer cells are nearly as efficient as the model ovarian granulosa cells (HGL5) in the uptake of cholesteryl esters and taxol from the lipid core of recombinant HDL (rHDL) complexes, indicating that HDL may be used to solubilize otherwise very poorly water soluble (hydrophobic) therapeutic agents for injection into the systemic circulation.

MATERIALS AND METHODS

Preparation of rHDL/drug complexes: Egg yolk phosphatidyl choline [Sigma] (1.8 mg in chloroform), 0.45 mg of unesterified cholesterol and 0.9 mg of cholesteroy oleate, both from Sigma, were dissolved in absolute alcohol, 300 µg of taxol (Mead Johnson) combined in a 15 ml conical glass tube and the solvent was evaporated under N_2. Next, 5 ml of buffer (10 mM Tris.HCl pH 8.0, 0.15 M NaCl, 1 mM EDTA 0.025% NaN_3) was added. The mixture was sonicated in a Branson sonicator using a microtip, under the stream of N_2 at 60°C for 15 min and then stopped for 5 min. This cycle was repeated 4 times. The temperature was then lowered to 40°C and 10 mg of apoA-I (*ZLB Bioplasma Inc.*, Berne Switzerland) was dissolved in 4 M guanidine.HCl and then added slowly (in 10 min) to the lipid dispersion. Sonication was resumed for another 30 min at 40°C under N_2 . The TX containing rHDL fractions were isolated by density gradient ultracentrifugation as described previously (22) and dialyzed overnight against 4.3 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4 at 4°C.

Determination of rHDL components. Total cholesterol (Data Medical Associates, Arlington TX), free cholesterol and phospholipids (Wako Pure Chemical Industries Ltd), were determined by respective enzymatic reagent kits, using microtiterplate assays (22). The concentration of cholesteryl esters was obtained as the difference between total and free cholesterol values. Taxol was determined by immunoassay as described by Leu et al (23). Protein determinations were carried out using Bradford reagent kits supplied by BioRad Laboratories.

Molecular weight determination. The molecular weight of the rHDL complex was determined by gel chromatography on 1.5X 80 cm AcA-34 (BioSepra/Life Technologies Inc.) columns using apoferritin, alcohol dehydrogenase, serum albumin and ovalbumin. The molecular weight of the rHDL complex was calculated based on the principles described by Ackers (24).

Cell cultures: The established cancer lines were obtained from ATCC and grown according to procedures provided by the ATCC, employing the respective recommended media, including 1% penicillin and streptomycin mixture and 10% fetal bovine serum (Gibco/Life Technologies). HGL5 cells (25), gift of Dr. William H. Rainey of Southwestern Medical Center, Dallas TX, were grown in DMEM F-12 (Gibco) with 1% ITS (BD Bio Sciences) and 10% FBS (Sigma). Cells were grown in 25 cm^2 flasks and split, using 0.25% trypsin to release the cells from the flasks, once 80-90% confluency was reached.

Monitoring of the uptake of core components from rHDL by cultured cells.

On the first day the cells were plated in 12 well plates (50000 cells/well) in their respective media. On the next day, the monolayers were washed with PBS pH 7.4 and then incubated at 37°C for 2 hrs with varying amount of rHDL-^{3}H-cholesteryl oleate and rHDL-Taxol (^{14}C -taxol) complexes

in serum free media (KGM). Following the 2 hr incubation period, the cells were washed with PBS pH 7.4 and dissolved in 0.5M NaOH in preparation for scintillation counting.

Screening of cell cultures for taxol uptake: Radio-labeled ^{14}C -taxol (Sigma) was incorporated into the rHDL particles along with the unlabeled taxol, as described above. The amount of TX taken up by the cells was monitored by scintillation counting. Taxol mass was computed from the specific radioactivities of the preparations that was earlier established by immunoassay (23).

Assessment of the cell killing potential of the HDL/drug complexes: The cytotoxic effect of the rHDL/drug preparations on cancer cells was assessed by sulforhodamine B (26). The assessment of TX toxicity was performed as follows. Cells grown to 80-90% confluency were detached from the flask by digesting with 0.25% Trypsin. The cells were subsequently washed from the flask with complete media and centrifuged for 5 minutes at 1000g to pellet the cells. The cells were then resuspended in complete media and an aliquot was diluted 1:1 with Trypan Blue. The cells were counted and their viability determined as follows: The cells seeded in 96 well microtiter plates (at 3000 cells per well) and allowed to attach for 24 hours. Taxol (Mead Johnson) was diluted to yield stock solutions of 10 nM to 100 nM and added in 50µl aliquots to each of the appropriate wells (total volume = 150 µl/well). Controls included media + TX. Following a 24 hr exposure to TX and TX/rHDL complexes respectively, the medium was removed and the cells were fixed with 100µl of 10% trichloro acetic acid for 1hr at 4°C. Subsequently, the samples were stained by 4% sulforhodamine B in 10% acetic acid. The stained protein was solubilized with 10mM Tris base and absorbance values were measured at 540 nm. using a Bio-Rad microtiterplate reader.

Western-blot Analysis: The cancer cells were grown until confluent, washed with PBS (pH 7.4) and scraped off the surface with 500µl of buffer containing 50mM Tris-HCl, 150mM NaCl, 0.02% sodium azide, 1% Triton X-100, 100µg/ml PMSF and 1µg/ml aprotinin. The cells were subsequently boiled for 5 mins and then centrifuged at 1,000 rpm for 10 mins and the supernatant used for western-blot analysis. Ten ug of protein was mixed with equal volume of sample buffer, boiled for 4 mins. and applied to a SDS-PAGE gel (10%). The protein was transferred to nitrocellulose overnight at 4^0C. Immunoreactive bands were detected with a polyclonal rabbit antiserum (Novus biologicals) raised against a peptide containing residues 496-509 from mouse SR-B 1, known to react with the human SRB1 analog, CLA-1. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody and visualization of immunoreactive bands was performed using the ECL™ detection system (Amersham).

RESULTS

Production and characterization of the rHDL / taxol complexes. Recombinant HDL complexes, containing taxol (rHDL/taxol) were prepared and characterized as follows. Table 1 shows the composition of the taxol containing rHDL complexes. The molecular weight of these rHDL/TX complexes (187,000) was established by gel chromatography as described under Methods. No loss of radioactivity was observed when a ^{14}C-taxol/rHDL preparation was rechromatographed on the same column, indicating that the rHDL preparation was stable and free from taxol leakage during processing. The molecular weight of the isolated rHDL appears somewhat large (187,000) for the amount of protein being present in the complex (~70% by weight). The 3 moles of taxol

per mole probably represent underloading of the rHDL/TX particles, as there is significant additional space in the core compartment, occupied by cholesteryl oleate (4% by weight).

The effectiveness of the rHDL / TX complex against cancer cells. The killing potential of the rHDL / drug complexes against tumor cells was assessed using the sulforhodamine B dye assay. Figure 1 shows the survival of PC-3 cells in the presence of taxol and rHDL/TX, respectively. The concentration of the drug was selected based on the studies of Liebmann et al (27). Similar studies conducted with DU145, MCF7 and OV1063 cell lines with essentially identical findings to those shown in Figure 1. These data show that the rHDL/TX complex is as efficient as TX in killing cancer cells even though the drug is apparently encapsulated by the HDL surface components.

The data in Table 2 show the uptake of HDL core components (^{3}H-cholesteryl oleate) and taxol from rHDL by an ovarian granulosa cell line (HGL5) and by a series of cancer cells. The ovarian HGL5 cell line was used as the control in these studies to compare the ability of cancer cells to incorporate HDL core components to that of the assumed normal physiological target (20-21). The data show that all cancer cells were able to efficiently incorporate cholesteryl esters from rHDL (>65% of the model cell line). The correlation (r=0.88) between the efficiency of cholesteryl ester and taxol uptake by a variety of malignant cells suggest that cancer cells express an HDL receptor, perhaps of the SR-B1 type, that allows the selective uptake of core components from HDL.

Figure 2 shows the immunoblots of cell extracts obtained with an SR-B1 antibody. Here the reactivity of the control ovarian cell line (HGL5) was compared with those of a number of cancer

cells and a fibroblast cell line. The data show significant immunoreactivity of all cancer cells, especially when compared to normal cells (fibroblasts) that are not known to express this receptor (28).

Finally the specificity of the core component uptake from rHDL was examined by assessing the competition between ^{3}H-cholesteryl oleate labeled and unlabeled rHDL preparations. The data for two cancer cell lines show (Figure 3) that the unlabeled rHDL suppressed the uptake of the labeled cholesteryl ester to a larger extent in the cancer cells compared to the HGL5 ovarian granulose cell line. These data suggest that the uptake of HDL core components is at least as specific (mediated by the SRB-1 type receptors) as for ovarian tissue.

DISCUSSION

Although improvement of cancer chemotherapy by the use of delivery vehicles has been impressive over the last several decades (4,5), important challenges, including toxicity of therapeutic agents and drug resistance encountered with several types of cancer. We have undertaken the study of rHDL complexes as potential delivery vehicles for chemotherapeutic agents because they appeared to have desirable properties as drug transporters, due to their size and the receptor mediated uptake of HDL core components (6,18,19). The latter feature could be particularly important as the rapidly dividing cancer cells are likely to have increased cholesterol requirements for membrane biogenesis. In fact, earlier studies have shown that the proliferation of adenocarcinoma cells (20) and breast cancer cells (21) was dependent on the uptake of HDL or HDL components. More recently, at least one breast cancer cell line was shown to express active HDL receptors (29). Based on these findings, combined with reports

in the literature (17-19), it is clear that HDL type particles may serve as highly efficient delivery vehicles of anticancer agents. Because of the relatively low levels of expression of these receptors in most peripheral tissues (23), it is likely that the drugs delivered via HDL type vehicles could have reduced toxic side effects compared to the free drug.

The application of rHDL particles as delivery vehicles for cancer chemotherapeutic agents may be advantageous for several reasons. Poor water solubility is a common feature of many pharmaceutical preparations including several chemotherapeutic agents (29,30). A number of recent efforts have been reported to overcome this problem and to reduce the toxicity of the intravenously administered taxol preparations (31,32). The rHDL/drug preparations may be particularly effective in this regard as the core components of HDL are normally made up of highly water insoluble compounds (triglycerides and cholesteryl esters). Concerning the effectiveness of taxol and other chemotherapeutic agents, the lack of tumor selectivity is another serious concern. The structure of rHDL allows for the alteration of lipid and protein composition and the covalent attachment of potential targeting vehicles to augment the selectivity of specific agents used in cancer chemotherapy.

Drug resistance of specific tumor cell lines is an another major concern during cancer chemotherapy. In addition to the known drug resistance tumors, certain malignant cells can develop resistance to a particular agent during chemotherapy. The ability of tumors to become resistant to chemotherapy has been linked to specific plasma membrane components (33), including the transmembrane P-glycoprotein (34). Because the rHDL preparation is able to interact with specific cell surface receptors (28) and deliver its core components to the interior

of the cells without endocytosis, this mechanism may be successful for delivering chemotherapeutic agents without inducing drug resistance.

In summary, we have prepared recombinant high density lipoprotein complexes (rHDL) that contain defined quantities of taxol. We have shown for the first time that the drug incorporated into the rHDL particles is efficiently taken up by several cancer cells presumably via a receptor mediated (SR-B1 type) mechanism. This drug delivery system may be effective in overcoming the poor solubility of many pharmaceutical preparations, increase the selectivity of anticancer drugs and possibly reduce the drug resistance against certain agents, encountered during chemotherapy.

Acknowledgements

This research was supported by grants from the Department of Defense Congressionally Directed Breast Cancer Research Program (BC5286) and the Bank One Cancer Research Fund, Institute for Cancer Research, University of North Texas Health Science Center.

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TABLES AND FIGURES: LACKO ET AL, 2002

TABLE 1. Composition of taxol containing rHDL preparations

Component	Mean weight (μg) per ml (n=4)	% Distribution	µMoles per ml	Moles per MW=187,000
Lecithin	428 ± 48	26	0.56	51
Cholesterol	9 ± 0.9	0.6	0.23	3
Cholesteryl Oleate	24 ± 1.6	1.5	0.035	4
ApoAI	1140 ± 99	70.5	0.042	5
Taxol	17 ± 1.9	1.1	0.02	3

Table 2. Comparison of the uptake of labeled (^{14}C) taxol and labeled (^{3}H) cholesteryl oleate from rHDL complexes by selected cancer cell lines. Uptakes by the non-malignant ovarian granulose cell line (HGL5) were used a the control values (100%)

Cell Line	Cellular uptake of core component (%)	
	^{14}C-Taxol	^{3}H-cholesteryl ester
HGL5	100	100
DU 145	67	75
MCF7	73	72
T47D	74.	90
OV 1063	74	75
PC3	83	91

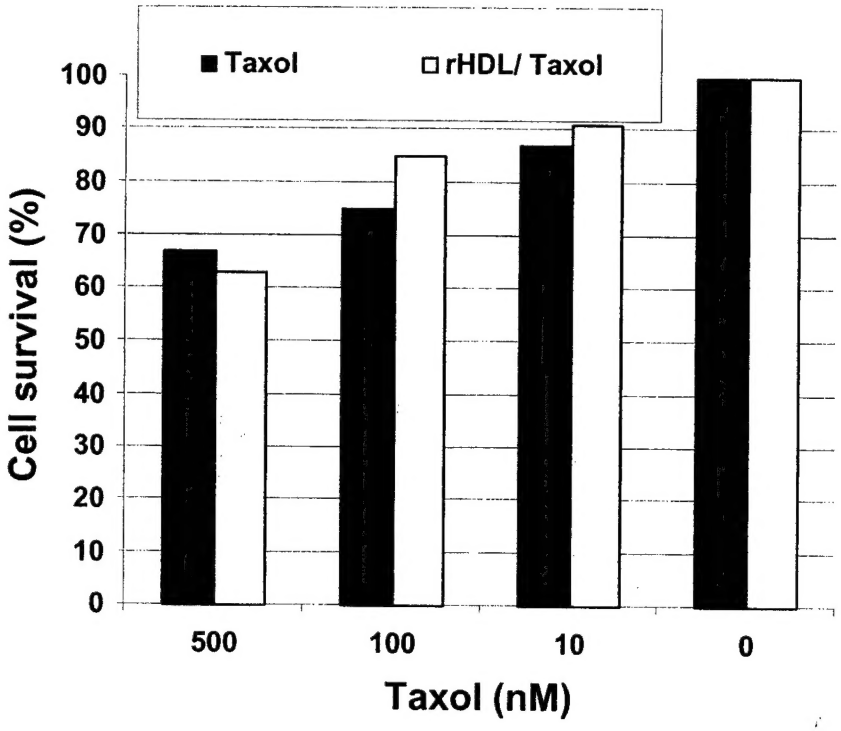

Figure 1. Comparison of the toxicity of taxol vs rHDL taxol preparations against PC-3 cells as monitored by the sulforhodamine dye assay.

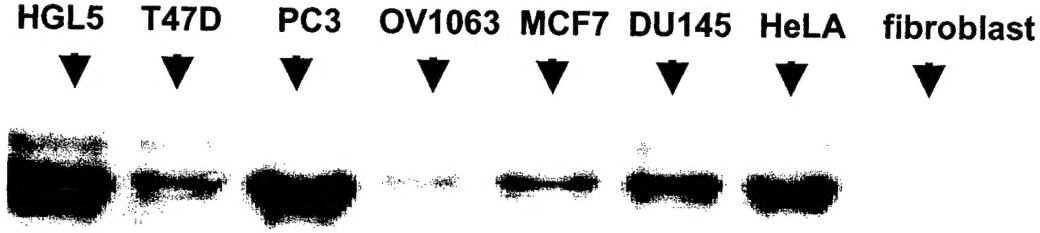

Figure 2. . Immunoblot of the SRB-1 receptor in normal and cancer cell lines

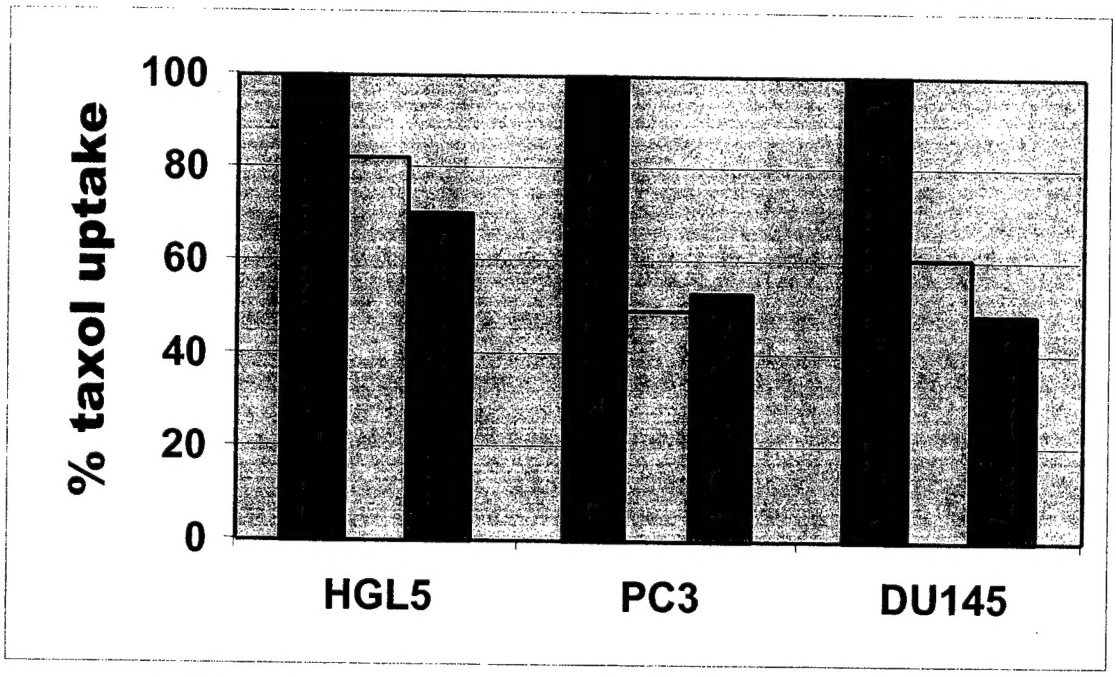

Figure 3. Comparison of the ^{3}H-cholesteryl ester uptake from rHDL preparations by ovarian granulosa (HGL5) and two cancer cell lines in the presence of half (dotted bars) and equal amounts of unlabeled rHDL (horizontally lined bars). The solid black bars represent the controls with no unlabeled HDL added. The data are presented as the percentage of the uptake by the HGL5 cells (100%).